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Frequent amplification of the c-*met* gene in scirrhous type stomach cancer*

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Summary Amplification of the c-*met* gene, that encodes hepatocyte growth factor receptor, was examined on human esophageal, gastric and colorectal carcinomas. Six (55%) of the 11 gastric carcinoma cell lines and 15 (23%) of the 64 advanced gastric carcinomas showed the c-*met* gene amplification. Among them, c-*met* amplification was detected in 5 gastric cancer cell lines, derived from scirrhous gastric carcinoma and in 5 (38%) of 13 scirrhous gastric carcinoma tissues. Furthermore, patients of gastric carcinoma with c-*met* amplification showed significantly advanced tumor stage and poorer prognosis than those without the amplification. Conversely, no amplification was detected in any of the esophageal and colorectal carcinoma cell lines as well as carcinoma tissues except one colonic carcinoma. These results overall suggest that amplification of the c-*met* gene might participate in carcinogenesis and progression of stomach cancer, especially scirrhous type stomach carcinoma.

A variety of human cancers expresses multi-autocrine loops of growth factor/receptor system such as EGF, TGF-α and TGF-β, which evidently play a crucial role in tumor progression [1] [2]. In gastric carcinomas, many growth factors are frequently overexpressed without gene amplification, while growth factor receptor type genes such as ERBB (EGF receptor), ERBB2 [3] [4] and K-*sam* (FGF receptor) [5] are often amplified. The amplification of the receptor genes closely correlates with tumor metastasis [4].

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Abbreviations: EGF, epidermal growth factor; TGF-α, transforming growth factor alpha; TGF-β, transforming growth factor beta; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor.

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An oncogene, *met*, was initially identified in NIH3T3 cells transfected with DNA from human osteosarcoma cell line (HOS) transformed with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [6] [7]. Subsequent analyses on proto-form of this gene revealed that 4.2kb of c-*met* cDNA encoded a receptor type tyrosine kinase with a molecular mass of 145KDa [8] [9]. Recent studies have shown that c-*met* product is a β-subunit of hepatocyte growth factor (HGF) receptor whose α-subunit is a 50KDa protein [10] [11]. These subunits are linked by two disulfide bonds to make an insulin receptor-like structure [12] [13]. Most recently, HGF has been found to be identical with scatter factor or lung fibroblast-derived mitogen [14] [15] [16].

In present study, we examined the c-*met* gene amplification in human esophageal, gastric and colorectal carcinomas, and compared with tumor stage and prognosis of the patients.

Materials and Methods

Cell culture and tissues. Eleven gastric carcinoma cell lines were obtained as follows. TMK-1 was established from poorly differentiated adenocarcinoma in our laboratory [17]. Five human gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. Suzuki (Fukushima Medical College, Fukushima). KATO-III cell line established from signet ring cell carcinoma was kindly provided by Dr. Sekiguchi (University of Tokyo, Tokyo). HSC-39 and HSC-43 cell lines established from scirrhous carcinoma were kindly provided from Dr. Yanagihara (Hiroshima University, Hiroshima) [18]. NTAS and NKPS cell lines also established from scirrhous carcinoma were kindly provided from Dr. Mai (Kanazawa University, Cancer Res. Inst., Kanazawa). Six esophageal carcinoma cell lines (TE-1, -4, -7, -8, -10, -12) were kindly provided by Dr. Nishihira (Tohoku University, Sendai). TCO was established from colon carcinoma in our laboratory [19]. Six colorectal carcinoma cell lines (WiDr, LoVo, DLD-1, CoLo320DM, CoLo201, SW837) were provided by the Japanese Cancer Resources Bank (JCRB). St-fib derived from fibroblast obtained from the stomach was established in our laboratory. They were routinely maintained in RPMI-1640 (Nissui Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS, Whittaker, Walkersville, MA) under the condition of 5% CO_2 in air at 37°C.

A total of 75 gastric carcinomas, 27 intestinal metaplastic mucosas of the stomach, 34 colorectal carcinomas and 5 esophageal carcinomas were used. Tumor tissues were obtained by surgical removal, frozen in liquid nitrogen and stored at -80°C. Corresponding non-neoplastic mucosa excluding muscle layer was taken from the same patient. We confirmed microscopically that tumor tissues mainly consisted of tumor cells and non-neoplastic mucosas did not contain tumor cells. In gastric carcinoma, histological classification and staging were made according to Japanese Research Society for Gastric Cancer [20].

Southern blot analysis. High-molecular-weight DNAs were prepared by the phenol-chloroform method [21]. DNAs were digested with *Taq*I, *Msp*I, *Hind*III, and 10μg of completely digested DNAs were electrophoresed on 0.8% agarose gel. After electrophoresis, DNAs were denatured, neutralized and then transferred to nitrocellurose filters. Filters were baked for 2h at 80°C under vacuum. Hybridization and washing were performed as described previously [22]. The filters were autoradiographed on Kodak XAR-5 films at -80°C.

Amplification of the c-*met* gene was defined as 3-fold or more increase of signal intensities than those of the corresponding non-neoplastic mucosa by densitometric tracing. Each case was confirmed with 3 separate experiments.

DNA probes. The 1.6kb p-*met*H DNA probe was provided by JCRB [23]. The 1.2kb p-*met*D was purchased from Oncor Inc. (Gaithersburg, MD). The g3 (D7S22) was provided from Dr.A. J. Jeffreys and Dr. A. Alves. The CRI-L1033 (D7S63) and The CRI-pS148 (D7S95) were purchased from Collaborative Research Inc. (Bedford, MA).

Results

Amplification of the c-*met* gene was examined in esophageal, gastric and colorectal carcinoma cell lines by Southern blot analysis with p-*met*H probe. As shown in Fig. 1, six (MKN-45, HSC-39, KATO-III, HSC-43, NKPS, and NTAS) out of the 11 gastric carcinoma cell lines had c-*met* amplification. MKN-45, established from poorly differentiated adenocarcinoma, showed 13.6-fold amplification, while the other 5 cell lines, all of which were originated from scirrhous carcinoma, showed 3 to 5-fold amplification of the c-*met* gene. St-fib, a fibroblast cell line derived from the gastric wall was used as a control to show a signal intensity from single copy of the c-*met* gene. No amplification of the c-*met* gene was detected in any of the six esophageal carcinoma cell lines nor the seven colorectal carcinoma cell lines (data not shown).

We also examined the alteration of chromosome 7q, on which c-*met* gene is located, on the 11 gastric carcinoma cell lines. In Southern blot analysis using p-*met*D, upstream probe of p-*met*H, the signal intensities were also magnified in the 6 cell lines with the gene amplification detected by p-*met*H probe (Fig. 1, middle panel). However, on another restriction fragment length polymorphism (RFLP) marker for chromosome 7q,

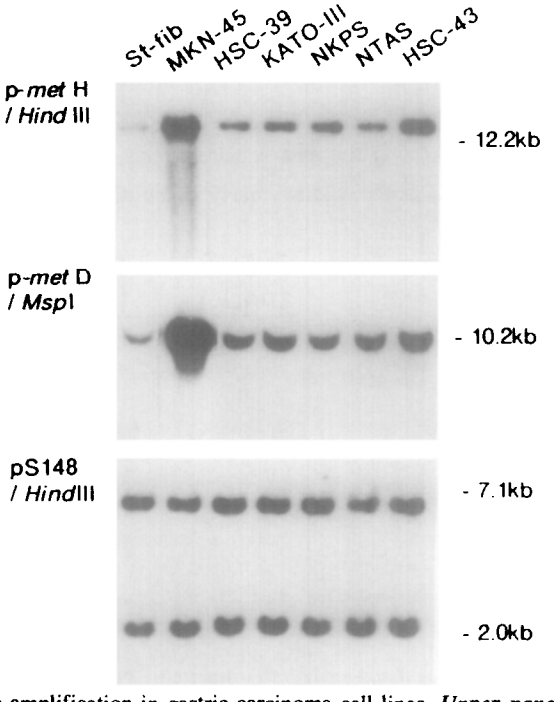

Fig. 1. c-*met* gene amplification in gastric carcinoma cell lines. *Upper panel*, Ten μg of *Hind*III-digested DNAs was analyzed by Southern blot using the probe p-*met*H. In MKN-45, HSC-39, KATO-III, NKPS, NTAS and HSC-43, the signal intensities by densitometry were magnified by 13.6-, 3.6-, 4.5-, 4.6-, 3.8-, and 5.5-fold, respectively, compared with that of St-fib. *Middle panel*, Southern blot analysis using the probe p-*met*D and *Msp*I-digested DNAs. *Lower panel*, the filter, examined by p-*met*H, was reprobed with the probe CRI-pS148, a RFLP marker for chromosome 7q.

Table 1. c-*met* gene amplification in gastrointestinal carcinomas

Histlogical type[a]		Examined cases	Amplification	Incidence of amplification
Gastric cancer				
Advanced [b]	well	26	5	19.2%
	poorly	25	5	20.0%
	scirrhous	13	5	38.5%
	total	64	15	23.4%
Early[b]		11	0	0
Intestinal metaplasia		27	0	0
Colorectal cancer		34	1	2.9%
Esophageal cancer		5	0	0

a)According to the criteria of the Japanese Research Society for Gastric Cancer [20]. Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; poorly, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; scirrhous, scirrhous gastric carcinoma.
b)Advanced carcinomas, invade beyond Tunica muscularis propria; early carcinomas, proliferation within Tela submucosa.

CRI-pS148, signal intensities on Southern blot filters were almost the same among all cell lines (Fig. 1, lower panel). From examination by other two RFLP marker for chromosome 7q, CRI-L1033 and g3, the same result was obtained (data not shown). These results indicated that alteration of ploidy on chromosome 7q did not occur in these cell lines.

Amplification of the c-*met* gene in the tumor tissues of the esophagus, stomach and colorectum is summarized in Table 1. Representative autoradiography is demonstrated in Fig. 2. The c-*met* gene amplification frequently occurred in gastric carcinoma tissues, whereas it was detected only in one of the 34 colorectal carcinomas and no esophageal carcinoma. Among the gastric carcinomas, 23% of the advanced carcinomas had amplified c-*met* gene, whereas early cases did not. Moreover,

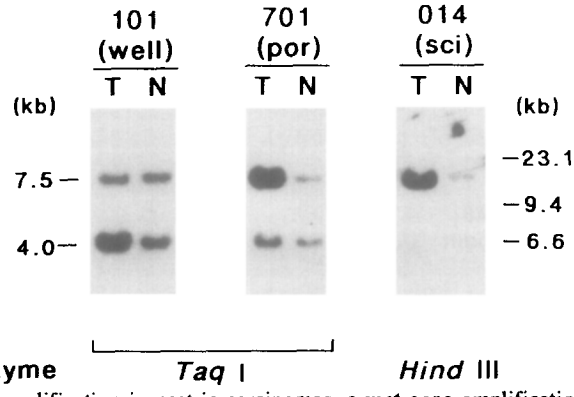

Fig. 2. c-*met* gene amplification in gastric carcinomas. c-*met* gene amplification was examined by Southern blot using *Taq*I- or *Hind*III-digested DNAs and the probe p-*met*H. Numbers above the lanes are sample numbers (T: tumor specimen, N: non-neoplastic mucosa). Magnification of the amplified gene was 4-, 9-, 14-fold at cases 101, 701, 014, respectively, by densitometric measurement. Histological types are according to the criteria of the Japanese Research Society for Gastric Cancer [20]. Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; por, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; sci, scirrhous gastric carcinoma.

amplification of the c-*met* gene was observed in 5 (38%) out of 13 scirrhous type gastric carcinomas.

Discussion

Among esophageal, gastric and colorectal carcinomas, amplification of the c-*met* gene was frequently associated with gastric carcinomas. The gene rearrangement accompanied with gene amplification was not found. In structural study on chromosome 7q using 5 RFLP markers, gene amplification was detected only on p-*met*H, 3'-end probe and p-*met*D, upstream probe of c-*met* gene [23]. No amplification was found in other three probes located at telomeric side of c-*met* gene on the long arm of chromosome 7. Therefore, c-*met* gene amplification occurred on relatively small locus of chromosome 7 without major rearrangement. Amplification of the c-*met* gene was found in one of paired alleles in all the cases. Another allele was not amplified or deleted. It had been reported that GTL-16, a gastric carcinoma cell line with the c-*met* gene amplification [13] has an amplicon including the c-*met* gene region without rearrangement nor mutation [24].

The most interesting finding in this study is that all the five gastric carcinoma cell lines established from scirrhous carcinoma and 38.5% of scirrhous carcinoma tissues showed the c-*met* gene amplification. Scirrhous type stomach cancer corresponds to diffusely infiltrating carcinoma or Borrmann's type IV carcinoma of the stomach showing vast fibrous stroma with rapid and extensive growth. Previous studies have shown the specific genetic features in scirrhous carcinoma. K-*sam* gene amplification preferentially occurs in this type of stomach cancer [5]. Simultaneous overexpression of PDGF and PDGFR mRNA is also detected in stomach cancers associated with plentiful fibrous stroma [25]. TGF-β and basic FGF are also overexpressed in scirrhous type stomach carcinomas [22] [26].

Since c-*met* product is the receptor protein for HGF [10] [11], gastric carcinoma cells with c-*met* amplification should be responsible for mitogenic [16] [27] [28] and motogenic [14] [15] properties of HGF. In view of recent evidence that HGF is mainly produced by fibroblasts [14] [15], HGF from fibroblast may bind to c-*met* protein on carcinoma cells, leading to DNA synthesis and migration of tumor cells in scirrhous carcinomas. In fact, we have confirmed that fibroblast cell line ST-fib obtained from the stomach expresses high level of HGF mRNA and then secrete a large amount of HGF into the culture media (data not shown).

As to clinical features, c-*met* amplification was closely related to tumor progression. That is, advanced gastric carcinomas (stage III and IV) shared frequent amplification of c-*met*, whereas stage I gastric carcinomas had no amplification. Furthermore, patients with c-*met* amplification showed significantly worse prognosis than those without the amplification (data not shown).

These results overall suggest that amplification of the c-*met* gene might be implicated in carcinogenesis and progression of stomach cancer, especially scirrhous type stomach carcinoma.

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